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(54) Title: EXPRESSION CASSETTE FOR PLANTS

(57) Abstract

The present invention relates to an expression cassette which can express a gene at high levels; to transformed bacterial and plant cells which contain the expression cassette; to transgenic plants which are produced from plant cells transformed with the expression cassette; and to a process of producing transgenic plants which exhibit a desired trait.

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EXPRESSION CASSETTE FOR PLANTS

FIELD OF INVENTION

The present invention relates to an improved expression cassette useful for expressing genes at high lev ls in transformed plants.

BACKGROUND OF THE INVENTION

Application of genetic engineering techniques to improve plant species can lead to plant species which exhibit increased resistance to pathogens, which display an increased tolerance to severe or erratic weather conditions and/or which are more nutritious. It is well established that the characteristics of pathogen tolerance, weather tolerance and nutritional composition are produced by the genetic makeup of the plant. Thus, introducing genetic material into a plant can bring about preferred characteristics and, accordingly, improved plant species.

The genetic factors which effect these desirable charactistics include genes for peptide products whose presence confers desired traits as well as those whose nucleotide sequences impede the production of undesirable proteins. Plants which have antipathogenic proteins present in the cells display increased resistance to pathogen challenge. Weather tolerance can be conferred by the production of certain proteins in plants. The nutritional value of plants is often related to the presence of proteins and, in particular, desirable amino acids which make them up. In addition to genes which encode peptides that effect the various characteristics, traits can be influenced by the presence of RNA molecules which are not translated. Thus, the introduction of genetic information can be used to provide or supplement desirable traits in plants.

Genetic information may be introduced into plants which will result in an increased resistance to pathogenic challenge. Specifically, the presence of antipathogenic proteins can increase resistance against viral infection as well as act as agents agianst bacteria and other microrganisms. Furthermore, peptides which act as pesticides, fungicides and herbicides may be introduced and expressed. In addition to introducing genetic sequences which encode such protein products, resistance to the same pathogenic agents may conferred on a plant by introducing genetic information which can be transcribed in the plant cell into RNA that will inhibit translation of RNA encoding proteins which are necessary in pathogen challenge.

Tolerance to varying and severe climate conditions can be conferred in plants by introduction of genetic information. The presence of various proteins can provide plants with the ability to adapt to and survive und r sub- ptimum climatic conditions. Genes which confer traits that through evolution have developed in plants that make them more adapted to survive cold, heat, drought or flooding for example, can be isolated. These genes can be introduced into plants which lack them and the plants will exhibit the phenotype the genes accord.

Similarly, the nutritional value of plants can be changed and increased through the introduction of genetic information. Genes encoding peptides and proteins comprised of amino acids which are desirable may be introduced into plants. The genes are expressed and the plant displays the improved composition accordingly.

The genes which are to be introduced into the plant may be derived from any source. Many genes which confer desirable traits in one species of plants may be transferred to confer the trait in a second, different plant. Antipathogenic genes may be derived from pathogens. Genes may be synthesized. Alternatively, the genes may be identical to those already found in the plant genome where they, by providing multiple copies of the genes, will be introduced in order to supplement and enhance the trait. Accordingly, heterologous as well as endogenous genes may be introduced into plants to confer or supplement a desired trait.

Methods to introduce genetic material into plants are widely known to those skilled in the art. Extensive work has been done with vectors of Agrobacterium tumerfaciens. Additionally, genetic material has been introduced into the genome of plants by impacting plant cells with microprojectiles coated with the genetic material. When the genetic material is introduced into the plant cells it can be incorporated into the plant's genome and if linked to the proper genetic regulatory elements, expressed. The expression of the introduced genes can confer traits on the host plant.

In order to achieve expression of introduced genetic material, the structural genes sought to be expressed must be linked to regulatory sequences. These regulatory sequences include a promoter, an initiation codon and a polyadenylation addition signal. Without these elements operably linked to the gene sought to be expressed,

expression cannot take place. Expression will take place when the genetic material to be introduced is constructed such that the gene to be expressed is downstream of the promoter and initiation codon, in proper reading frame with the initiation codon and upstream from the polyadenylation addition signal.

Higher levels of expression are often desirable and can be acheived with the addition of genetic sequences in addition to those minimally required. Untranslated regions flanking the promoter can often lead to a higher level of gene expression. Similarly, untranslated regions flanking the initiation codon can also result in a higher level of expression. In addition, the sequences flanking the poly A addition signal may lead to more efficient processing of the RNA transcribed and gene expression accordingly.

Expression of the coat protein of tobacco mosaic virus, alfalfa mosaic virus, cucumber mosaic virus, and potato virus X in transgenic plants has resulted in plants which are resistant to infection by the respective virus. In order to produce such transgenic plants, the coat protein gene must be inserted into the genome of the plant. Furthermore, the coat protein gene must contain all the genetic control sequences necessary for the expression of the gene after it has been incorporated into the plant genome.

The present invention relates to an improved expression cassette for introducing desired genes into plants. This cassette comprises a promoter, an AT rich 5' untranslated region, an initiation region comprising the sequence AAXXATGG, a gene and a poly(A) addition signal which contains untranslated flanking regions. According to the present invention, genes which are introduced into plants will be expressed at high levels because the genetic regulatory sequences controlling expression facilitate such high expression.

INFORMATION DISCLOSURE

European patent application EP 0 223 452 describes plants that are resistant to viral diseases and methods for producing them. The process described comprises the steps of transforming a plant with a DNA insert comprising a promoter, a DNA sequence derived from the virus, and a poly(A) addition sequence.

PCT patent application PCT/US86/00514 refers to a method of conferring resistance to a parasite to a host of the parasite.

An et al. (1985) "New cloning vehicles for transformation of

higher plants", EMBO J. 4:277-285 describe the construction of an expressi n plasmid which may be stably replicated in both E. coli and A. tumerfaciens.

An, G. (1986) "Development of plant promoter xpression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells", Plant Physiol. 81:86-91, reports vast differences in promoter activities of transferred genes within the same cells as well as in independently derived cell lines.

Kozak, M. (1986) "Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes", Cell 44:283-292, discloses the optimal sequence around the ATG initiator codon of the preproinsulin gene for initiation by eukaryotic ribosomes.

Loesch-Fries et al. (1987) "Expression of alfalfa mosaic virus RNA 4 in transgenic plants confers virus resistance", EMBO J 6:1845-1851, disclose that expression of the coat protein gene of alfalfa mosaic virus in transgenic plants confers resistance to infection by the virus.

Mazur, B. J. and Chui, C.-F. (1985) "Sequence of a genomic DNA clone for the small subunit of ribulose bis-phosphate carboxylase-oxygenase from tobacco", Nucleic Acids Research 13:2373-2386, disclose the DNA sequence of the small subunit of ribulose bis-phosphate carboxylase-oxygenase from tobacco.

Olson, M. K. et al (1989) "Enhancement of heterologous polypeptide expression by alterations in the ribosome-binding-site sequence", J. Biotech. 9:179-190, discloses the increase in gene expression of heterologous genes in E. coli due to the presence of an AT-rich 5' untranslated region.

Pietrzak et al. (1986) "Expression in plants of two bacterial antibiotic resistant genes after protoplast transformation with a new plant expression vector", Nucleic Acids Research 14:5857-5868, disclose expression in plants of foreign genes introduced into the plant using an expression vector containing a movable expression cassette consisting of the Cauliflower mosaic virus 35S promoter and transcription terminator seperated by a polylinker containing several unique restriction sites.

Powell-Abel et al. (1986) "Delay of disease development in

transgenic plants that express the tobacco mosaic virus coat protein gene", Science 232:738-743, disclose increased resistance toward infection by tobacco mosaic virus in transgenic plants containing the coat protein gene from tobacco mosaic virus.

Tumer et al. (1987) "Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants", EMBO J. 6:1181-1188, disclose transgenic tobacco and tomato plants transformed with the coat protein gene of alfalfa mosaic virus display increased resistance to infection by alfalfa mosaic virus.

SUMMARY OF THE INVENTION

The present invention relates to an expression cassette which can express a desired gene at high levels. The present invention relates to an expression vector which comprises an expression cassette. The high level expression vector of the present invention comprises: a promoter; a 5' untranslated region which is at least 60% A and T; an initiation codon comprising Kozak's element; a cloning site where a desired gene may be inserted to form a functional expression unit; and a 3' untranslated region which comprises a poly(A) addition signal and flanking sequence which yields high level expression. The present invention relates to transformed bacterial and plant cells which contain the expression vector. The present invention relates to transgenic plants which are produced from plant cells transformed with the expression vector. The present invention relates to a process of producing transgenic plants with desirable traits by producing the plants from plant cells which have been transformed with an expression vector which contains gene conferring such traits.

DETAILED DESCRIPTION OF THE INVENTION

Certain conventions are used in Charts 1-9 to illustrate plasmids and DNA fragments as follows:

(1) The single line figures represent both circular and linear double-stranded DNA.

(2) Asterisks (*) indicate that the molecule represented is circular. Lack of an asterisk indicates the molecule is linear.

(3) Junctions between natural boundaries of functional components are indicated by vertical lines along the

horizontal lines.

(4) Genes or functional components are indicated below the horizontal lines.

(5) Restriction sites are indicated above the horizontal lines.

(6) Distances between genes and restriction sites are not to scale. The figures show the relative positions only unless indicated otherwise.

(7) The following abbreviations are used to denote function and components:

a) P_{ca} = CaMV35S promoter;

b) I_c = CMV intergenic region, the intergenic region comprising the initiation codon and AT rich 5' untranslated region;

c) S_{ca} = CaMV35S poly(A) addition signal; and

d) Nos = Nos nptII gene.

Most of the recombinant DNA methods employed in practicing the present invention are standard procedures, well known to those skilled in the art, and described in detail in, for example, European Patent Application Publication Number 223452 published November 29, 1986, which is incorporated herein by reference. Enzymes are obtained from commercial sources and are used according to the vendor's recommendations or other variations known in the art. General references containing such standard techniques include the following: R. Wu, ed. (1979) Methods in Enzymology, Vol. 68; J. H. Miller (1972) Experiments in Molecular Genetics; T. Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual; D. M. Glover, ed. (1985) DNA Cloning Vol. II; H.G. Polites and K.R. Marotti (1987) "A step-wise protocol for cDNA synthesis". Biotechniques 4:514-520; S.B. Gelvin and R.A. Schilperoort, eds. Introduction, Expression, and Analysis of Gene Products in Plants, all of which are incorporated by reference.

For the purposes of the present disclosure the following definitions for terms used herein are meant to apply.

"Expression cassette" means a DNA fragment which contains a gene operably linked to regulatory sequences necessary for gene expression.

"Promoter" means a promoter which is functional in the host plant.

"Initiation region" includes the initiation codon and nucleotides flanking the initiation codon.

"Operably linked" refers to the linking of nucleotide regions encoding specific genetic information such that the nucleotide regions are contiguous, the functionality of the region is preserved and will perform relative to the other regions as part of a functional unit.

"AT rich 5'" untranslated region is a nucleotide sequence is composed of at least 60% adenine or thymine nucleotides.

"Untranslated flanking region" refers to nucleotide sequences which are 3' of the termination codon and end at the poly(A) addition signal.

"Vector" is a vehicle by means of which DNA fragments can be introduced into host organisms.

"Expression vector" is a vehicle by means of which DNA fragments that contain sufficient genetic information can be introduced into host organisms and can, therefore, be expressed by the host.

"Antipathogenic gene" is a gene which encodes a DNA sequence which is either the antisense sequence of a pathogenic gene or the antipathogenic gene encodes a peptide whose presence in an organism confers an increased resistence to a pathogen.

To practice the present invention, the gene sought to be introduced into the plant's genetic material must be inserted into a vector containing the genetic regulatory sequences necessary to express the inserted gene. Accordingly, a vector must be constructed to provide the regulatory sequences such that they will be functional upon inserting a desired gene. When the expression vector/insert construct is assembled, it is used to transform plant cells which are then used to regenerate plants. These transgenic plants carry the gene in the expression vector/insert construct. The gene is expressed in the plant and the trait which the gene influences will be conferred upon the plant.

In order to express the gene, the necessary genetic regulatory sequences must be provided. Both transcription and translation signals are necessary for gene expression once the gene is transferred and integrated into a plant genome. It must, therefore, be engineered to contain a plant expressible promoter, a translation initiation codon (ATG) and a plant functional poly(A) addition signal

(AATAAA) 3' of its translation termination codon.

In the present invention, additional genetic regulatory sequences are provided in order to btain high levels f gene expressi n. As described above, an xpression vector must contain a promoter, an intiation codon and a p ly(A) additi n signal. In order to get a higher level of expression, untranslated regions 5' and 3' to the inserted genes are provided. Furthermore, certain sequences flanking the initiation codon optimize expression. The promoter used is one that is chosen for high level expression.

A 5' untranslated region which results in higher level expression of an inserted gene is provided downstream from the promoter and upstream from the initiation codon. This region contains at least 60% of the sequence as Adenine and Thymine. There is a statistical bias for expression when such an AT rich region is located between the promoter and intiation codon. This preference is utilized in the preferred embodiment of the present invention by inclusion of an AT rich 5' untranslated region intermediate of the promoter and initiation codon.

The present invention also contains a specific nucleotide sequence flanking the initiation codon. This preferred sequence, termed Kozak's element, is AAXXATGG wherein X represents any of the four nucleotides. The presence of the initiation codon following Kozak's rule results in higher level expression when used in an expression vector. In the preferred embodiment of the present invention, the small subunit of ribulose bis-phosphate carboxylase-oxygenase (SS RUBISCO) contains an initiation codon in which Kozak's element is used.

Furthermore, the present invention contains a 3' untranslated region downstream from the cloning site where the coat protein gene is inserted and upstream from the poly(A) addition signal. The sequence of this 3' untranslated region results in a statistical bias for protein production. The sequence promotes high level expression. The poly(A) addition signal is found directly downstream from the 3' untranslated region and can be derived from the same source. In the preferred embodiment of the present invention, the 3' untranslated region and poly(A) addition signal are derived from CaMV 35S gene or the phaseolin seed storage protein gene.

The poly(A) addition signal from CaMV, nopaline synthase,

octopine synthase, bean storage protein, and SS RUBISCO genes are also suitable for this construction. Several promoters which function in plants are available, but the best promoters are the constitutive promoter from cauliflow r mosaic virus (CaMV, a plant DNA virus) and the SS RUBISCO gene.

Using methods well known to those having ordinary skill in the art, plant cells are transformed with the vector construct and the plant cells are induced to regenerate. The resulting plants contain the coat protein genes and produce the coat protein. The production of the protein confers upon the plant an increased resistance to infection by the virus from which the coat protein gene was derived.

In addition, the expression vector of the present invention comprises a DNA molecule comprising a promoter, an AT rich 5' untranslated region, an initiation region, a gene and a polyadenylation signal with untranslated flanking regions wherein the promoter is upstream and operably linked to the AT rich 5' untranslated region, the AT rich 5' untranslated region is upstream and operably linked to the initiation region which is upstream and operably linked to the antipathogenic gene which is upstream and operably linked to the poly(A) addition signal.

The DNA molecule described above may comprise a promoter derived from the Cauliflower mosaic virus CaMV 35S gene. The DNA molecule comprising the expression vector described above may contain an AT-rich 5' untranslated region is derived from the 5' untranslated region of Cucumber mosaic virus CMV coat protein gene or SS RUBISCO gene. Similarly, the initiation region in the DNA molecule described above may be derived from the 5' untranslated region of Cucumber mosaic virus CMV coat protein gene or SS RUBISCO gene. The initiation region of the DNA molecule described above comprises the DNA sequence AAXXATGG. Furthermore, the DNA molecule described above may comprise a poly(A) addition signal derived from either the Cauliflower mosaic virus CaMV 35S gene; the phaselin storage protein gene, the nopalinesynthase gene, the octopine synthase gene, the bean storage protein gene or the SS RUBISCO gene.

The expression vector described above may comprise a DNA molecule in which the antipathogen gene is a viral pathogen coat protein gene, a viral enzyme gene, a gene derived from a host gene or an unrelated gene. Furthermore, the introduced gene may be for a

peptide which acts as a pesticide, a fungicide or a herbicide when expressed in a plant. The gene may encode a peptide whose presence confers tolerance to severe or eradic climatic conditions. The gene, alternatively, may encode a protein whose presence in a plant enhances the nutritional value of the plant. The present invention provides a recombinant DNA molecule which comprises any desired gene operably linked to genetic regulatory elements necessary for high expression in transformed plants. Moreover, the gene may not be required to be transcribed into a peptide. The gene may encode an antisense strand to a nucleotide sequence forwhich inhibition of translation is desirable.

The expression vector may be an Agrobacterium derived binary vector. The expression vector may be used to transformed cells and transgenic plants may be produced comprising the transformed cells.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Example 1 Isolation of WMVII RNA.

Watermelon mosaic virus II (WMV II) was propagated in zucchini squash (Cucurbita pepo L) plants and RNA was isolated by the method described by Yeh and Gonsalves (Virology 143:260, 1985).

Example 2 Isolation of PRV-p RNA.

Papaya ringspot virus strain prv (PRV-p) was propagated in jelly melon, Cucumis metuliferus (Nand.) Mey. Acc. 2549 plants and RNA was isolated by the method described by Yeh and Gonsalves (Virology 143:260, 1985).

Example 3 Isolation of ZYMV RNA.

Zucchini yellow mosaic virus (ZYMV) was propagated in zucchini squash (Cucurbita pepo L) plants and RNA was isolated by the method described by Yeh and Gonsalves (Virology 143:260, 1985).

Example 4 Synthesis of Double-stranded cDNA.

The procedure used to make double stranded cDNA from isolated viral RNA is the same for all viral RNA isolated above. The purified RNA was subjected to the cDNA synthesis protocol described by Polites and Marotti (Biotechniques 4:514, 1986) and because this RNA contains an A-rich region at its 3'-end (similar to that found for many eukaryotic mRNAs) the procedure was straight-forward. The synthesis of double stranded cDNA was also done as described by Polites and Marotti. After double-stranded cDNA was synthesized, it was purified by passage through a G-100 S phadex column, precipitated with

ethanol, and suspended in 20 μl of 10X EcoRI methylase buffer (100 mM NaCl, 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 80 μM S-adenosyl methionine, and 100 μg/ml bovine serum albumin). An additional amount of S-adenosyl methionine (1 μl of a 32 mM solution) was added to the reaction mixture, followed by the addition of 1 μl (20 units) EcoRI methylase. The reaction was incubated at 37°C for 30 minutes and stopped by incubation at 70°C for 10 minutes. Then 1 μl (5 units) of E. coli DNA polymerase I Klenow fragment was added and incubated at 37°C for 10 minutes, followed by phenol/chloroform extraction and ethanol precipitation. The pellet was washed in 70% ethanol, then 70% ethanol/0.3 M sodium acetate. The pellet was dried and resuspended in 8 μl of 0.5 μg/μl phosphorylated EcoRI linkers (Collaborative Research, Inc., 128 Spring St., Lexington, MA 02173). One μl of 10X ligase buffer (800 mM Tris-HCl ph 8.0, 200 mM $MgCl_2$. 150 mM DTT, 10 mM ATP) and 1 μl of T4 DNA ligase (4 units) were added, and the reaction was incubated overnight at 15°C. The ligation reaction was stopped by incubation at 65°C for 10 minutes. Sixty μl of H_2O, 10 μl of 10X EcoRI salts (900 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 100 mM NaCl), and 10 μl of EcoRI (10 units/μl) were added, and the reaction was incubated at 37°C for 1 hour. The reaction was stopped by phenol/chloroform and chloroform extractions. The reaction mixture was then size fractionated by passage through a Sephadex G-100 column and the fractions containing the largest double stranded cDNA molecules were concentrated by butanol extractions, precipitated with ethanol, and resuspended in 10 μl of H_2O. Five μl of the double stranded cDNAs was added to 0.5 μg of pUC19 DNA (which had been previously treated with phosphatase to remove the 5' phosphates), 1 μl of 10X ligase buffer, and 1 μl of T4 ligase, and the reaction was incubated at 15°C for 16 hours. The resulting ligated pUC19-coat protein gene double stranded cDNA molecules were transformed into E.coli host cells as described by the manufacturer (Bethesda Research Laboratories, Inc., Gaithersburg, MD 20877) and plated on medium containing 50 μg/ml ampicillin, 0.04 mM IPTG, and 0.004% X-Gal. Bacterial colonies showing no blue color were selected for further analysis. Clones containing the 3'-region and possibly the coat protein gene were identified by hybridization against a ^{32}P-labeled oligo-dT. Bacterial colonies showing hybridization to this probe should contain at least the poly(A) region of the particular poty-

virus genome. Several of the hybridizing bacterial clones were selected and plasmid DNAs were isolated according to methods known to those skilled in the art.

Example 5 Identification of the PRV-p Coat Protein Gene.

Several of the cloned cDNAs of PVP-p RNA were sequenced by the chemical DNA sequencing method described by Maxam and Gilbert (Methods of Enzymology 65:499, 1980). Based on this information and comparative analysis with other potyviruses clone number pPRV-117 was found to contain a complete copy of the PRV-p coat protein gene. The N-terminus of the coat protein was identified by the location of the dipeptide sequence Gln-Ser. The length of the PRV-p coat protein gene coding region is consistent with a gene encoding a protein of about 33 kDal.

Example 6 Construction of a Plant-Expressible PRV-p Coat Protein Gene Cassette with CaMV 35S Promoter and Polyadenylation Signal and CMV 5' Untranslated Region and Translation Initiator ATG.

Attachment of the necessary plant regulatory signals to the PRV-p coat protein gene was accomplished by constructing a translational fusion with a clone originally designed for the expression of the CMV coat protein gene, using clone pUC1813/CP19. Plasmid pUC1813/CP19 is a vector suitable for agrobacterium mediated gene transfer. An EcoRI-EcoRI fragment was removed from pDH51/CP19 and placed into the EcoRI site of the plasmid, pUC1813 (available from Robert K., Department of Chemistry, Washington State University, Pullman, Washington), creating plasmid pUC1813/CP19. Plasmid pUC1813/CP19 was described in U.S. patent application Serial Number 07/135,591 filed on December 21, 1987 incorporated herein by reference. This translational fusion clone was constructed by first identifying two restriction enzyme sites within clone pUC1813/CP19. One site (Tth111 I) is located between amino acids 13 to 17 while the other site (BstX I) is located near the end of the 3'-untranslated region of the CMV coat protein gene.

Addition of these specific restriction enzyme sites to the PRV-p coat protein gene was accomplished by the polymerase chain reaction technique, using an instrument and Taq polymerase purchased from Perkin Elmer-Cetus, Emeryville, Ca. Specifically, two respective 5' and 3' oligomers (CGACGTCGTCAGTCCAAGAATGAAGCTGTG, containing a Tth111 I site and (CCCACGAAAGTGGGGTGAAACAGGGTCGAGTCAG, containing a BstX I

site), sharing at least 20 nucleotides with the PRV-p coat protein gene were used to prime synthesis and gen amplification of the coat protein gene. After synthesis, the amplified fragments were digested with Tth111 I and BstX I to expose the sites.

As shown in Chart 1, pUC1813/CP19 is the expression vector which contains the CMV coat protein gene. Plasmid pUC1813/CP19 contains Tth111 I and BstX I sites.

The digested, amplified fragments are ligated into the respective exposed sites of pUC1813/CP19 and the expected new construction was identified using methods known to those skilled in the art. Polymerase chain reaction techniques were used to amplify PRV-P coat protein gene containing the Tth111I and BstXI sites. The plasmid pUC1813/CP19 and PRV-P coat protein gene fragments were digested with Tth111I and BstXI and ligated to each other. The resulting clone, designated pUC1813/CP19-PRVexp, was subjected to nucleotide sequencing to ensure that the cloning and gene amplification did not introduce any detrimental artifacts. The sequence showed no artifacts, suggesting that this plant expression cassette should be capable of expressing a PRV-p coat protein gene which contains an additional 16 amino acids of CMV coat protein at its N-terminus.

Example 7 Construction of a Micro T-DNA Plasmid Containing the Plant-expressible PRV-p Coat Protein Gene Construction.

As depicted in Chart 2, the plant expression cassette for the PRV-p coat protein gene was transferred into a suitable micro T-DNA vector which contains the necessary Agrobacterium T-DNA transfer signals for transfer from an Agrobacterium and integration into a plant genome, and a wide host-range origin of replication (for replication in Agrobacterium). Plasmid pUC1813/CP19-PRVexp was digested with Hind III and the resulting 2.2 kb insert fragment containing the plant-expressible cassette was removed and ligated into the Hind III site of the modified Agrobacterium-derived micro-vector pGA482 (modification included the addition of the β-glucuronidase gene). The micro T-DNA vector, pGA482, is available from G. An, Institute of Biological Chemistry, Washington State University, Pullman, WA. The resulting plasmid was designated, pGA482/G/CP19-PRVexp and is shown in Chart 2. This plasmid (or derivatives thereof) was transferred into virulent or avirulent strains of Agrobacterium tumefaciens or rhizogenes, such as A208, C58, LBA4404,

C58Z707, A4RS, A4RS(pRiB278b), and others. Strains A208 C58, LBA4404, and A4RS are available from American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD. Bacteria A4RS (pRiB-278b)is available from Dr. F. Cass -Delbart, C.N.R.A., Rout de Saint Cyr. F78000, Versailles, France. Strain C58Z707 is available from Dr. A.G.Hepburn, Dept. of Agronomy, University of Illinois, Urbana, IL.

After transfer of the engineered plasmid pGA482/G/CP19-PRVexp into any of the above listed Agrobacterium strains, these Agrobacterium strains can be used to transfer and integrate within a plant genome the plant-expressible PRV-p coat protein gene contained within its T-DNA region. This transfer can be accomplished using the standard methods for T-DNA transfers which are known to those skilled in the art, or this transfer can be accomplished using the methods described in a U.S. patent application Serial Number 07/135,655, filed on December 21, 1987, entitled "Agrobacterium Mediated Transformation of Germinating Plant Seeds" and incorporated herein by reference.

Example 8 Construction of a Plant-expression Cassette for Expression of Various Genes in Transgenic Plants.

In the preferred embodiment of the present invention, the following expression cassette was constructed to provide the necessary plant regulatory signals (which include the addition of a promoter, 5' untranslated region, translation initiation codon, and polyadenylation signal) to the gene inserts in order to achieve high level expression of the inserts. The expression cassette may be used to express any genes inserted therein. Accordingly, the applicability of the expression cassette goes beyond its use in expressing coat protein genes. Rather, the expression cassette may be used to express any desired protein in transgenic plants. The expression cassette is the preferred expression system for expressing viral coat protein genes in plants.

The expression cassette of the preferred embodiment contains: a constitutive promoter; a 5' untranslated region which enhances gene expression; an initiation codon which comprise Kozak's element; a cloning site where the gene to be expressed may be inserted to produce a functional expression unit; and a 3' untranslated region which comprises a poly(A) addition signal and untranslated flanking

regions which result in a higher level of expression.

More specifically, the expression cassette which is the preferred embodiment of the present invention consists of the cauliflower mosaic virus (CaMV) 35S transcript promoter, the 5'-untranslat d region of cucumber mosaic virus (CMV), the CMV translation initiation codon, and the CaMV polyadenylation signal. The construction of this expression cassette utilized the Polymerase Chain Reaction (PCR) technique to obtain correct position of the plant regulatory signals and the addition of convenient restriction enzyme sites which allow for the easy addition of a coat protein gene and the excision of the completed cassette so it can be transferred to other plasmids.

To accomplish the construction of this expression cassette the following oligomers were synthesized:

1. 5'-GAAGCTTCCGGAAACCTCCTCGGATTCC-3', contains a HindIII site at its 5'-end and contains 21 bases which are identical to 21 bases in the 5'-flanking region of CaMV.

2. 5'-GCCATGGTTGACTCGACTCAATTCTACGAC-3', contains a NcoI site at its 5'-end which contains a translation initiation codon which conforms to Kozak's rules and has 21 bases which are identical to 21 bases in the antisense strand of the CMV 5'-untranslated region.

3. 5'-GCCATGGTTGCGCTGAAATCACCAGTCTC-3', contains a NcoI site at its 5'-end (which contains the same translation initiation codon as oligomer 2) and has 20 bases which are identical to 20 bases in the 3'-untranslated region of CaMV.

4. 5'-GAAGCTTGGTACCACTGGATTTTGGTT-3', contains a HindIII site at its 3'-end and has a 20 base match with the flanking DNA region 3' of the CaMV polyadenylation site (on the antisense strand).

These oligomers were used to amplify sequences contained within the CMV expression clone referred to as pUC1813/CP19, shown in Chart 1, and referred to above. As depicted in Chart 3, the PCR technique was used to amplify the gene regulatory regions in pUC1813/CP19. Amplification of the 5'-flanking, CMV 5'-untranslated region, and CMV initiation codon (which was modified to conform to Kozak's rule AAXXATGG) resulted in a fragment of about 400 base pairs in length and amplification of the CaMV 3-untranslated and flanking regions resulted in a fragment of about 200 base pairs in length. These fragments were digested with NcoI and HindIII, isolated from a polyacrylamide gel, and then ligated into HindIII digested and

phosphatase treated pUC18. The resulting clone is referred to as p18CaMV/CMV-exp and is shown in Chart 3.

Example 9 Identification of the WMVII C at Protein Gene

The cloned WMVII cDNA insert from clone pWMVII-41-3.2 which was produced as described above, was sequenced by using both the chemical (Maxam and Gilbert, Methods of Enzymology 65:499, 1980) and enzymatic (Sanger et al., Proc. Natl. Acad. Sci. U.S.A. 74:5463, 1977) methods. Based on this information and comparative analysis with other potyviruses, the nucleotide sequence of clone pWMVII-41-3.2 was found to contain a complete copy of the WMVII coat protein gene. The N-terminus of the coat protein was suggested by the location of the dipeptide sequence Gln-Ser. The length of the WMVII coat protein gene coding region (281 amino acids) is consistent with a gene encoding a protein of about 33 kD.

Example 10 Construction of a Plant-expressible WMVII Coat Protein Gene Cassette with CaMV 35S Promoter and Polyadenylation Signal and CMV Intergenic Region and Translation Initiator ATG.

As depicted in Chart 4, attachment of the necessary plant regulatory signals to the WMVII coat protein gene was accomplished by using the PCR technique to amplify the WMVII coat protein gene using oligomers which would add the necessary sites to its 5′ and 3′ sequences. Following this amplification the resulting fragment is digested with the appropriate restriction enzyme and cloned into the NcoI site of the above described expression cassette containing plasmid, p18CaMV/CMV-exp. Clones containing the WMVII coat protein gene insert need only be checked to determine correct orientation with respect with the CaMV promoter. However, to ensure that no artifacts have been incorporated during the PCR amplification the entire coat protein gene region is checked by nucleotide sequence analysis.

To obtain the amplified WMVII coat protein gene with NcoI restriction enzyme sites on both ends the following two oligomers were synthesized:

1. 5′-ACCATGGTGTCTTTACAATCAGGAAAAG-3′, which adds a NcoI site to the 5′-end of the WMVII coat protein gene and retains the same ATG translation start codon which is present in the expression cassette, p18CaMV/CMV-exp.

2. 5′-ACCATGGCGACCCGAAATGCTAACTGTG-3′, which adds a NcoI site

to the 3'-end of the WMVII coat protein gene, this site can be ligated into the expression cassette, p18CaMV/CMV-exp.

The cloning of this PCR WMVII coat protein gene, using these two oligomers, into p18CaMV/CMV-exp yields a plant expressibl WMVII gene (referred to as p18WMVII-exp) which, following transcription and translation, will generate a WMVII coat protein which is identical to that derived from the WMVII coat protein gene nucleotide sequence. However, this coat protein will differ, because of necessary genetic engineering to add the ATG initiation codon and by including the last four amino acids of the 54 kD nuclear inclusion protein (which is adjacent to the Glu-Ser protease cleavage site); the amino acids added are Val-Ser-Leu-Glu-N-ter WMVII. The addition of these four amino acid residues should not affect the ability of this coat protein to yield plants which are resistant to WMVII infections, because the N-terminal region of potyvirus coat proteins appear not to be well conserved for either length or amino acid identity. However, if this is found to be a problem its replacement would involve the use of a different oligomer to obtain N-terminal variations of the WMVII coat protein gene. The cloned construction of the plant expressible WMVII coat protein gene is referred to as p18WMV-II-exp, and is shown in Chart 4.

Example 11 Construction of a Micro T-DNA Plasmid Containing the Plant-expressible WMVII Coat Protein Gene Construction.

As depicted in Chart 5, the plant expression cassette for the WMVII coat protein gene was transferred into a suitable micro-T-DNA vector which contains the necessary Agrobacterium T-DNA transfer signals (to mediated transfer from an Agrobacterium and integration into a plant genome) and wide-host range origin of replication (for replication in Agrobacterium) to form plasmid pGA482/G/CPWMVII-exp. To construct this plasmid, plasmid p18WMVII-exp was digested with Hind III (which cuts within the polycloning sites of pUC18, well outside of the expression cassette), and an 1.8 kb fragment containing the plant-expressible cassette was removed and ligated into the Hind III site of the modified Agrobacterium-derived micro-vector pGA482 (modification included the addition of the β-glucuronidase gene). The micro T-DNA vector, pGA482, is shown in Chart 2 and available from G. An, Institute of Biological Chemistry, Washington State University, Pullman, WA. The resulting plasmid was designated,

pGA482/G/CPWMVII- xp is shown in Chart 5. This plasmid (or derivatives thereof) was transferred into virulent or avirulent strains of Agrobacterium tumefaciens or rhizogenes, such as A208, C58, LBA4404, C58Z707, A4RS, A4RS(pRiB278b), and others. Strains A208 C58, LBA4404, and A4RS are available from American Type Culture Collecti n (ATCC), 12301 Parklawn Drive, Rockville, MD. Bacteria A4RS(pRiB-278b)is available from Dr. F. Casse-Delbart, C.N.R.A., Routede Saint Cyr. F78000, Versailles, France. Bacteria C58Z707 is available from Dr. A.G.Hepburn, Dept. of Agronomy, University of Illinois, Urbana, IL.

After transfer of the engineered plasmid pGA482/G/CPWMVII-exp into any of the above listed Agrobacterium strains, these Agrobacterium strains can be used to transfer and integrate within a plant genome the plant-expressible WMVII coat protein gene contained within its T-DNA region. This transfer can be accomplished using the standard methods for T-DNA transfers which are known to those skilled in the art, or this transfer can be accomplished using the methods described in U.S. Patent application SN 07/135,655 filed December 21, 1987 entitled "Agrobacterium Mediated Transformation of Germinating Plant Seeds". In addition, it has recently been shown that such Agrobacteria are capable of transferring and integrating their T-DNA regions into the genome of soybean plants. Thus these strains could be used to transfer the plant expressible WMVII coat protein gene into the genome of soybean to develop a soybean plant line which is resistant to infection from soybean mosaic virus strains.

Example 12 Microprojectile Transfer of pWMVII-exp into Plant Tissues.

Recently an alternative approach for the transfer and integration of DNA into a plant genome has been developed. This technique relies on the use of microprojectiles on which the DNA (plasmid form) is attached. These microprojectiles are accelerated to high velocities and their momentum is used to penetrate plant cell walls and membranes. After penetration into a plant cell the attached DNA leaches off the microprojectile and is transferred to the nucleus where DNA repair enzymes integrate the "free" DNA into the plant genome. In its present form the process is entirely random, but plant tissues which have been successfully transformed by the plasmid DNA (or part of it) can be identified and cultured to homogeneity by

the use of selectable marker genes (such as the bacterial neomycin phosphotransferase II gene, NPTII), or reporter genes (such as the bacterial beta-glucuronidase gene, Gus). Successful use of particle acceleration to transform plants has recently been shown for soybean and the transfer of p18WMVII-exp into the genome could result in obtaining soybean plants which are resistant to infections from soybean mosaic virus strains.

The use of this process for the transfer of p18WMVII-exp can be accomplished after the addition of either plant expressible genes NPTII or Gus genes or both. Plasmids that have the nptII and Gus genes to p18WMVII-exp are shown in Chart 6, and referred to as p18GWMVII-exp and p18NGWMVII-exp. In addition, the construction described in Example 11 can also be used for microprojectile transfer as it already has both the nptII and Gus genes attached to the pWMVII-exp cassette (see Chart 5). The only difficulty which the use of pGA482GG/cpWMVII-exp may encounter during transfer by the micro-projectile process is due to its large size, about 18kb, which may have a lower efficiency transfer and such larger plasmid generally yield less DNA during propagation.

To construct plasmid p18GWMVii-exp, plasmid p18WMVii-exp is digested with BamHI and ligated with a 3.0 kilobase BamHI isolated fragment containing the Gus gene. To construct plasmid p18NGWMVii-exp, the plasmid p18GWMVii-exp is digested with SmaI and ligated with a 2.4 kb isolated fragment containing the Nos-nptII gene generated by digestion with DraI and StuI.

Example 13 Identification of the ZYMV Coat Protein Gene.

The cloned ZYMV cDNA insert from clone pZYMV-15, which was cloned using the method described above, was sequenced by using both the chemical (Maxam and Gilbert, Methods of Enzymology 65:499, 1980) and enzymatic (Sanger et al., Proc. Natl. Acad. Sci. U.S.A. 74:5463, 1977) methods. Based on this information and comparative analysis with other potyviruses the nucleotide sequence of clone pZYMV-15 was found to contain a complete copy of the ZYMV coat protein gene. The N-terminus of the coat protein was suggested by the location of the dipeptide sequence Gln-Ser which is characteristic of cleavage sites in potyviruses (see Dougherty et al. EMBO J. 7:1281, 1988). The length of the ZYMV coat protein gene coding region (280 amino acids) is consistent with a gene encoding a protein of about 31.3 kD.

Example 14 Construction f a Plant-expressible ZYMV Coat Protein Gene Cassette with CaMV 35S Promoter and Polyadenylation Signal and CMV Intergenic Region and Translation Initiator ATG.

As depicted in Chart 7, attachment of the necessary plant regulatory signals to the ZYMV c at protein gene was accomplished by using the PCR technique to amplify the ZYMV coat protein gene using oligomers which would add the necessary sites to its 5' and 3' sequences. Following this amplification the resulting fragment is digested with the appropriate restriction enzyme and cloned into the NcoI site of the above expression cassette containing plasmid, pUC18CP-exp. Clones containing the ZYMV coat protein gene insert need only be checked to determine correct orientation with respect with the CaMV promoter. However, to ensure that no artifacts have been incorporated during the PCR amplification the entire coat protein gene region is checked by nucleotide sequence analysis.

To obtain the amplified ZYMV coat protein gene with NcoI restriction enzyme sites on both ends the following two oligomers were synthesized:

1. 5'-ATCATTCCATGGGCACTCAACCAACTGTGGC-3', which adds a NcoI site to the 5'-end of the ZYMV coat protein gene and retains the same ATG translation start codon which is present in the expression cassette, pUC18cpexp.

2. 5'-AGCTAACCATGGCTAAAGATATCAAATAAAGCTG-3', which adds a NcoI site to the 3'-end of the ZYMV coat protein gene, this site can be ligated into the expression cassette, pUC18cpexp.

The cloning of this PCR ZYMV coat protein gene, using these two oligomers, into pUC18cpexp yields a plant expressible ZYMV gene (referred to as pUC18cpZYMV) which following transcription and translation will generate a ZYMV coat protein which is identical to that derived from the ZYMV coat protein gene nucleotide sequence. However, this coat protein will differ, because of necessary genetic engineering to add the ATG initiation codon followed by Gly, which is the amino acid 3' adjacent to the Ser of the polyprotein cleavage site. The Gly amino acid residue was selected for the potential N-terminal amino acid because many potyvirus coat proteins have either an Ser, Gly, or Ala at their N-terminal. However, if this is found to be a problem its replacement would involve the use of a different oligomer to obtain a different N-terminal amino acid for the ZYMV

coat protein. The cloned construction of the plant expressible ZYMV coat protein gene is referred to pUC18cpZYMV, and is shown in Chart 7.

Example 15 Construction of a Micro T-DNA Plasmid Containing the Plant-expressible ZYMV Coat Protein Gene Construction.

Following the teachings of Example 11 with appropriate modifications, the construction of a micro T-DNA plasmid containing a plant-expressible ZYMV coat protein was constructed. Plasmid pUC18cpZYMV (Chart 7) was digested with Hind III (which cuts within the poly-cloning sites of pUC18, well outside of the expression cassette), and a 1.6 kb fragment containing the plant-expressible cassette was removed and ligated into the Hind III site of the micro-vector pGA482 (Chart 2). The resulting plasmid was designated, pGA482GG/cpZYMV is shown in Chart 8.

After transfer of .the engineered plasmid pGA482GG/cpZYMV into Agrobacterium strains, the Agrobacterium strains can be used to transfer and integrate within a plant genome the plant-expressible ZYMV coat protein gene contained within its T-DNA region.

Example 16 Microprojectile Transfer of pUC18cpZYMV into Plant Tissues.

Following the teachings of Example 12, the microprojectile transfer technique can be used to introduce the ZYMV coat protein gene with appropriate genetic regulatory sequences into plant tissues.

The use of this process for the transfer of pUC18cpZYMV can be accomplished after the addition of either plant expressible genes NPTII or Gus genes or both. Plasmids that have the nptII and Gus genes to pUC18cpZYMV are shown in Chart 9 and referred to as pUC18GcpZYMV and pUC18NGcpZYMV. In addition, the construction described in Example 15 can also be used for microprojectile transfer as it already has both the nptII and Gus genes attached to the pUC18cpZYMV cassette (see Chart 8). The only difficulty which the use of pGA482GG/cpZYMV may encounter during transfer by the microprojectile process is due to its large size, about 18kb, which may have a lower efficiency transfer and such larger plasmid generally yield less DNA during propagation.

To construct plasmid pUC18GCPZYMV, plasmid pUC18CPZYMV is digested with BamHI and ligated to a 3.0 BamHI isolated fragment

which contains the Gus gene. To construct plasmid pUC18GCPZYMV, plasmid pUC18GCPZYMV is digested with SmaI and ligated with a 2.4 kb isolated fragment containing the Nos nptII gene isolated by digestion with DraI and StuI.

CHARTS

CHART 1

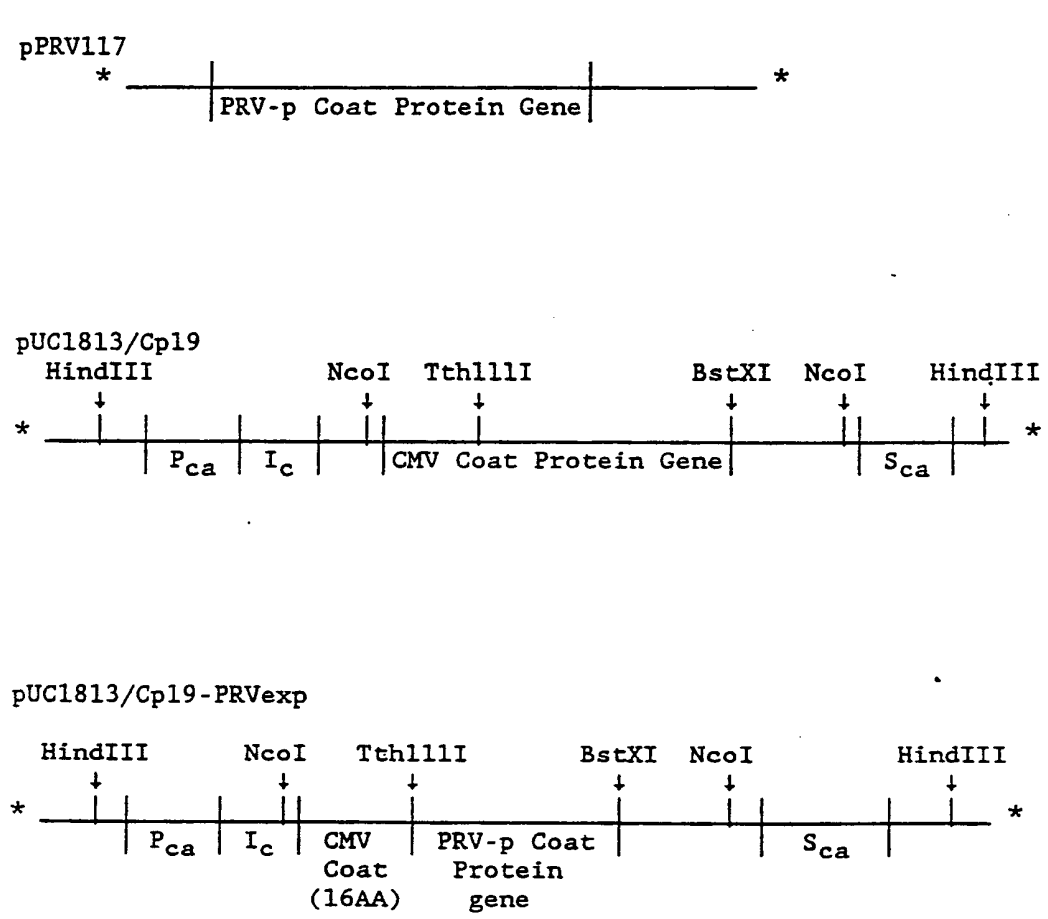
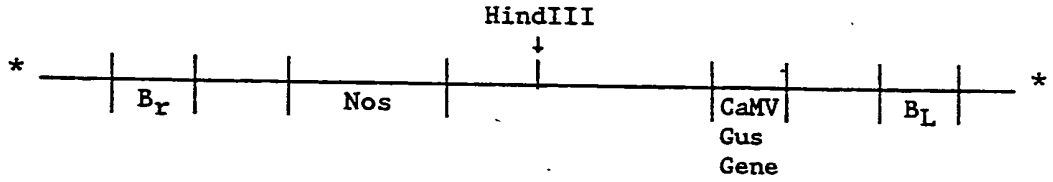


CHART 2

pGA482



pGA482/G/CP19-PRVexp

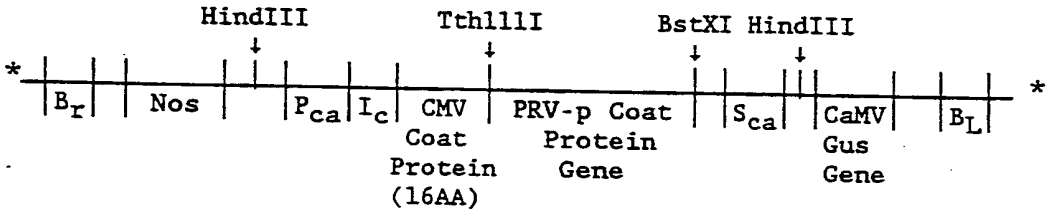
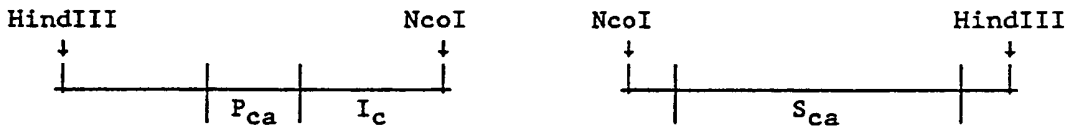


CHART 3



p18CaMV/CMV-exp

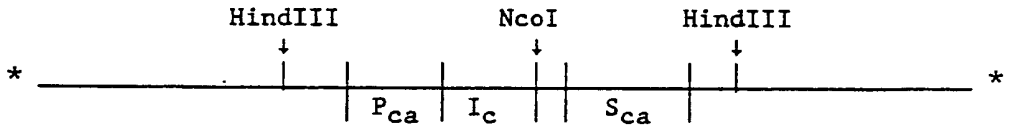
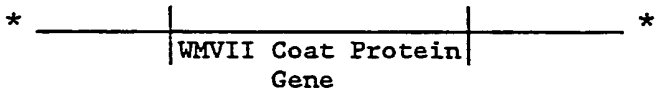
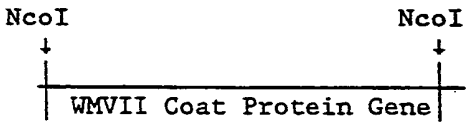


CHART 4

pWMVII-41-3.2



PCR Generated Gene



p18WMVII-exp

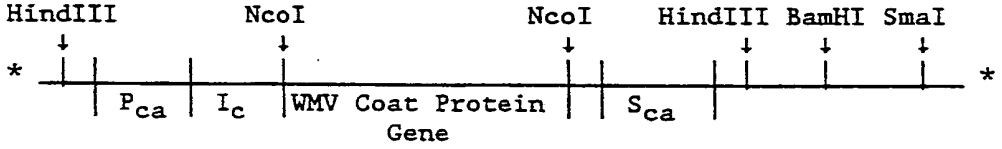


CHART 5

pGA482/G/CPWMVII-exp

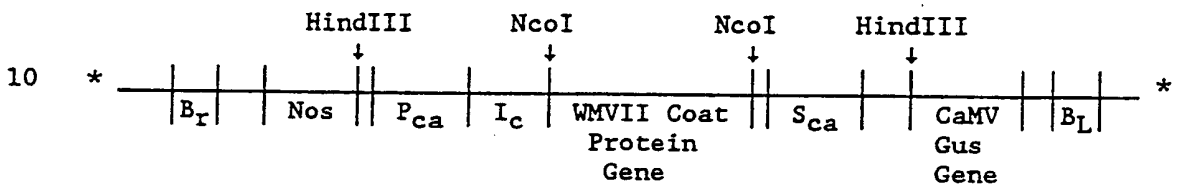
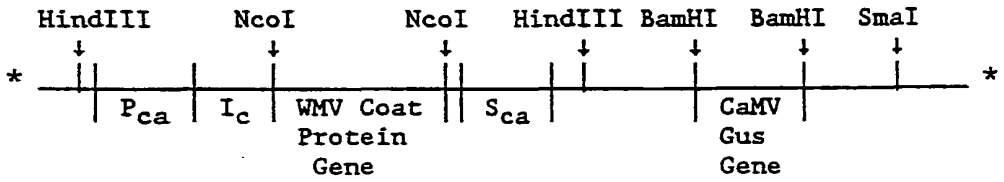


CHART 6

p18GWMVII-exp



p18NGWMVII-exp

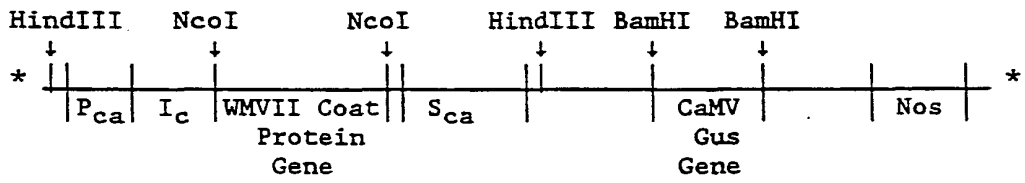
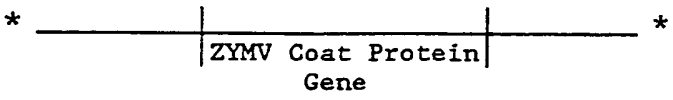
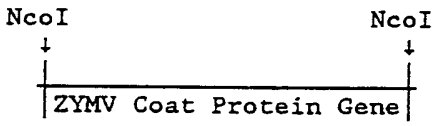


CHART 7

pZYMV-15



ZYMV Coat Protein Gene



pUC18CpZYMV

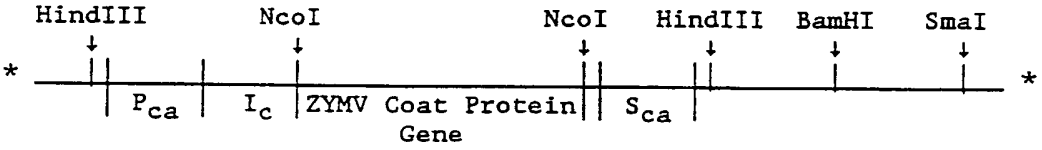


CHART 8

pGA482/GG/cpZYMV

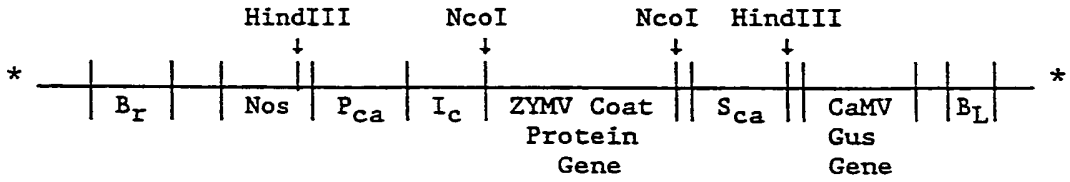
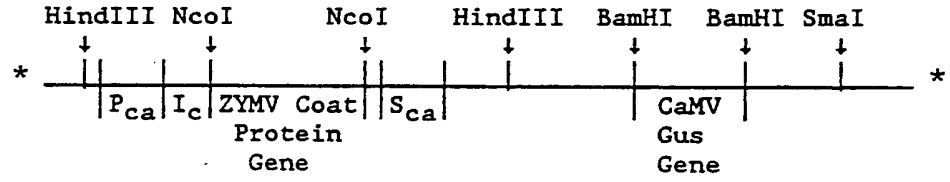
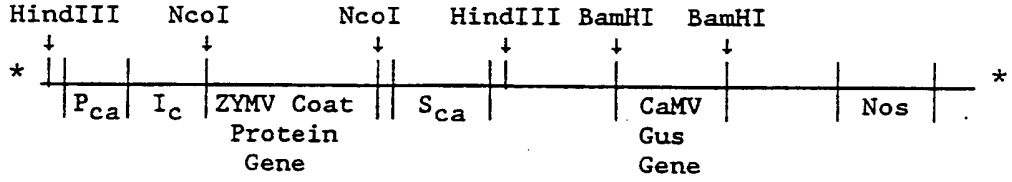


CHART 9

pUC18GCpZYMV



pUC18NGCpZYMV



CLAIMS

1. A DNA plant expression cassette comprising:

a) a promoter;

b) an AT rich 5' untranslated region;

c) an initiation region, said initiation region comprising the sequence AAXXATGG;

d) a gene; and

e) a poly(A) addition signal; said poly(A) addition signal containing untranslated flanking sequences;

wherein said promoter is upstream from said AT rich region; said AT rich region is upstream from said initiation region; said initiation region is upstream and operably linked to said DNA molecule encoding a gene, and said gene is upstream and operably linked to said poly(A) addition signal.

2. A DNA plant expression cassette according to Claim 1 wherein said promoter is Cauliflower mosaic virus CaMV 35S promoter.

3. A DNA plant expression cassette according to Claim 1 wherein said AT-rich 5' untranslated region is derived from the 5' untranslated region selected from the group consisting of the 5' untranslated region of Cucumber mosaic virus CMV coat protein gene and the 5' untranslated region of SS RUBISCO gene.

4. A DNA plant expression cassette according to Claim 1 wherein said initiation region is derived from the 5' untranslated region selected from the group consisting of the 5' untranslated region of Cucumber mosaic virus CMV coat protein gene and the 5' untranslated region of SS RUBISCO gene.

5. A DNA molecule according to Claim 1 wherein said poly(A) addition signal is selected from the group consisting of: the poly(A) signal derived from Cauliflower mosaic virus CaMV 35S gene; the poly(A) signal derived from phaseolin storage protein gene; the poly(A) signal derived from nopalinesynthase gene; the poly(A) signal derived from octopine synthase gene; the poly(A) signal derived from bean storage protein gene; and, the poly(A) signal derived from SS RUBISCO.

6. A DNA plant expression cassette according to Claim 2 wherein;

a) said AT rich 5' untranslated region and said initiation region are derived from the 5' untranslated region of Cucumber mosaic virus CMV coat protein gene; and

b) said poly(A) addition signal is derived from Cauliflower mosaic virus CaMV 35S gene.

7. A DNA plant expression cassette according to Claim 1 wherein said gene is an antipathogenic gene.

8. A DNA plant expression cassette according to Claim 6 wherein said gene is an antipathogen gene.

9. A DNA plant expression cassette according to Claim 7 wherein said pathogen derived gene is a coat protein gene.

10. A DNA plant expression cassette according to Claim 8 wherein said pathogen derived gene is a coat protein gene.

11. A transformed plant cell containing a DNA plant expression cassette according to Claim 1.

12. A transformed plant cell according to Claim 11 wherein said DNA plant expression cassette comprises:

a) a Cauliflower mosaic virus CaMV 35S promoter;

b) an AT rich 5' untranslated region and initiation derived from the 5' untranslated region of Cucumber mosaic virus CMV coat protein gene; and,

c) a poly(A) addition signal derived from Cauliflower mosaic virus CaMV 35S gene.

13. A transgenic plant comprising transformed plant cells according to Claim 11.

14. A transgenic plant according to Claim 13 wherein said DNA plant expression cassette comprises:

a) a Cauliflower mosaic virus CaMV 35S promoter;

b) an AT rich 5' untranslated region and initiation derived from the 5' untranslated region of Cucumber mosaic virus CMV coat protein gene; and,

c) a poly(A) addition signal derived from Cauliflower mosaic virus CaMV 35S gene.

15. A transgenic plant according to Claim 13 selected from the group consisting of the families Cucuribitaceae, Caricaceae, Solanaceae, and Leguminose.

16. A transgenic plant according to Claim 15 wherein said DNA plant expression cassette comprises:

a) a Cauliflower mosaic virus CaMV 35S promoter;

b) an AT rich 5' untranslated region and initiation derived from the 5' untranslated region of Cucumber mosaic virus CMV coat protein gene; and,

c) a poly(A) addition signal derived from Cauliflower mosaic virus CaMV 35S gene.

17. A process for introducing a trait in a plant comprising the steps of:

a) isolating a gene whose expression in a plant confers said trait;

b) constructing a DNA plant expression cassette according to Claim 1 with the isolated gene;

c) transforming plant cells with said DNA plant expression cassette; and

d) regenerating plants from said transformed plant cells.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/03095

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/82, C 12 N 5/14, A 01 H 4/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N, A 01 H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
O,X	UCLA Symposia, New Ser., vol. 62, Plant Gene Systems and Their Biology, 1987, Alan R. Liss, Inc., C. Dean et al.: "Expression of petunia rbcS gene fusions in transformed tobacco plants", pages 289-300, see the whole article	1,3-5,11, 13,17
X	Mol. Gen. Genet., vol. 212, no. 3, June 1988, Springer-Verlag, J.D.G. Jones et al.: "Expression of bacterial chitinase protein in tobacco leaves using two photosynthetic gene promoters", pages 536-542, see the whole article	1,3-5,11, 13,17
Y		2,6-10,12, 14-16
Y	WO, A, 87/07644 (DIATECH LTD) 17 December 1987, see page 8, paragraph 4; pages 33-36,39	1-17

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th October 1989	06. 12. 89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	The EMBO Journal, vol. 6, no. 1, January 1987, IRL Press Ltd (Eynsham, Oxford, GB), H.A. Lütcke et al.: "Selection of AUG initiation codons differs in plants and animals", pages 43-48, see the abstract	1-17
A	Plant Physiology, vol. 85, 1987, K.A. Barton et al.: "Bacillus thuringiensis delta-endotoxin expressed in transgenic Nicotiana tabacum provides resistance to lepidopteran insects",. pages 1103-1109, see figure 1; page 1105, right-hand column, paragraphs 3,4	1-17
A	Nucleic Acids Research, vol. 14, no. 8, 1986, IRL Press Ltd (Oxford, GB), N.E. Tumer et al.: "The genes encoding the small subunit of ribulose-1,5-bis-phosphate carboxylase are expressed differentially in petunia leaves", pages 3325-3342, see figure 2	1-17
A	Nucleic Acids Research, vol. 15, no. 8, 1987, IRL Press Ltd (Oxford, GB), D.R. Gallie et al: "The 5'-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts in vitro and in vivo", pages 3257-3273, see page 3270, para-graph 1	1-17
O,A	Journal of Cellular Biochemistry, UCLA Symposia on Molecular & Cellular Biology, Abstracts of the 17th Annual Meetings, Molecular Biology of Plant-Pathogen Interactions, 28 February - 10 April 1988, Supplement 12C, 1988, Alan R. Liss, Inc., (New York, US), H. Quemada et al.: "The nucleotide sequence of cDNA clones from RNA 3 of cucumber mosaic virus strains C and WL", page 287, abstract no. Y 333, see the abstract	3,4,12,14

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8903095 SA 29946

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/11/89
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		EP-A- 0270611	15-06-88
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